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Replication of adenovirus and SV40 chromosomes *in vitro*

By T. J. Kelly, P. J. Rosenfeld, R. J. Wides, E. A. O'Neill, J. J. Li and M. S. Wold

Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, *U.S.A.*

As an approach to studying the mechanisms involved in the replication of eukaryotic chromosomes, we have developed and characterized cell-free replication systems for the animal viruses, adenovirus and SV40. In this report we summarize recent work on the proteins required for the initiation of DNA synthesis in these two systems. The adenovirus origin of DNA replication was shown to consist of three functionally distinct sequence domains. Cellular proteins that specifically recognize each of these domains were purified and characterized. Initiation of adenovirus DNA replication was reconstituted from two virus-encoded and three cell-encoded factors. The SV40 origin of replication consists of a 65 base pair DNA segment that contains a high affinity binding site for the viral initiation protein T antigen. Evidence is presented that the first step in initiation of SV40 DNA replication involves the specific binding of T antigen to the origin, followed by the local unwinding of the two strands of the template. The unwinding reaction is specific for DNA templates containing the SV40 origin and requires ATP hydrolysis. In addition to T antigen, efficient unwinding requires a cellular factor(s) that can be replaced by the single-stranded DNA binding protein of *Escherichia coli*. These results indicate that the recently discovered helicase activity of T antigen plays a central role in initiation of viral DNA synthesis.

Introduction

The mechanisms involved in the replication of eukaryotic chromosomes are not yet understood. By analogy with prokaryotic systems it seems likely that analysis of the replication of the simple chromosomes of animal viruses will generate information that will be useful in defining such mechanisms. Our approach to this problem has been to develop cell-free systems that are capable of replicating exogenous viral DNA templates *in vitro*. We have previously described cell-free replication systems for two well-characterized viruses, namely adenovirus and SV40 (Challberg & Kelly 1979; Li & Kelly 1984).

The adenovirus genome is a linear, duplex DNA molecule of about 35000 base pairs. The extreme termini of the viral genome have identical nucleotide sequences and contain the viral origins of DNA replication (Tamanoi & Stillman 1983; Challberg & Rawlins 1984; Rawlins *et al.* 1984; Guggenheimer *et al.* 1984; de Vries *et al.* 1985; Leegwater *et al.* 1985; Wides *et al.* 1987). Studies of the replication process *in vivo* have demonstrated that initiation of DNA synthesis takes place at the two ends of the viral genome with about the same frequency (for review see Kelly (1984)). After each initiation event, a daughter strand is synthesized in the $5' \rightarrow 3'$ direction displacing one of the two parental strands. Upon completion of the first daughter strand, the displaced parental strand serves as template for the synthesis of a second daughter strand. *In vitro* studies have resulted in the identification of three viral proteins that participate in the replication process: the 80 kDa preterminal protein (Challberg *et al.* 1980),

the 140 kDa adenovirus DNA polymerase (Enomoto *et al.* 1981; Lichy *et al.* 1982), and the 72 kDa single-stranded DNA binding protein (van der Vliet & Levine 1973). The preterminal protein plays a central role in initiation of viral DNA replication (see below). The Ad DNA polymerase participates both in initiation and in elongation of nascent DNA chains, whereas the single-stranded DNA binding protein is required only during chain elongation. In addition to the virus-encoded proteins, several cellular proteins are required for optimal DNA replication *in vitro* (Nagata *et al.* 1982, 1983; Rawlins *et al.* 1984; Rosenfeld *et al.* 1987). Recent work on the identification and characterization of these cellular proteins is summarized below.

The papovavirus SV40 appears to be an excellent model for a single chromosomal replicon (for review see DePamphilis & Wasserman (1982)). The viral genome is a 5.2 kilobase circular duplex DNA molecule that is assembled into a minichromosome in infected cells. DNA replication is initiated within a unique origin on the viral chromosome and proceeds bidirectionally (Danna & Nathans 1972; Fareed *et al.* 1972). The initiation reaction requires a specific interaction between sequence elements in the origin and the viral initiation protein, T antigen (Wilson *et al.* 1982; Shortle *et al.* 1979; Myers & Tijian 1980). The elongation of nascent DNA chains appears to be mediated largely by cellular replication proteins. SV40 DNA replication in the cell-free system appears to mimic closely viral DNA replication *in vivo* (Li & Kelly 1984, 1985; Stillman *et al.* 1985; Li *et al.* 1986*a*, *b*; Murakami *et al.* 1986; Smale & Tjian 1986). Recent analysis of the system by biochemical methods has led to the identification of some of the proteins required for DNA replication and has provided a better understanding of the mechanisms involved in initiation, elongation and segregation.

Results

(*a*) *Adenovirus DNA replication* in vitro

(i) *The adenovirus origin of DNA replication*

The initiation of adenovirus DNA replication takes place at the ends of the linear viral genome and occurs by a novel mechanism in which a virus-encoded protein (the preterminal protein or pTP) serves as the primer for DNA synthesis. The fundamental initiation reaction is the formation of a phosphodiester linkage between dCMP, the first residue in the new daughter strand, and a serine residue in the pTP (Challberg *et al.* 1980). The formation of this dCMP–pTP initiation complex is dependent upon the presence of specific nucleotide sequence elements at the termini of the parental genome and requires the participation of both viral and cellular replication proteins. Once the complex has formed, elongation of the new daughter strand proceeds by addition of nucleotides to the free 3′OH group of the dCMP residue that is covalently bound to the pTP.

We have made use of the cell-free adenovirus DNA replication system to explore the sequence requirements for initiation. For this purpose we measured the relative initiation efficiency *in vitro* of linear templates containing deletion and base substitution mutations. The data (summarized in figure 1) indicate that the adenovirus origin of DNA replication contains at least three functionally distinct domains that contribute to the overall efficiency of the initiation reaction. Domain A consists of the first 18 base pairs of the viral genome and represents the minimal origin of replication. Domains B (nucleotides 19–40) and C (nucleotides

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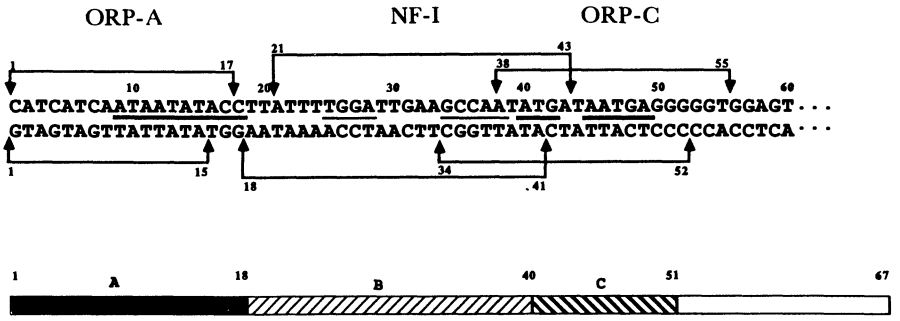

FIGURE 1. Summary of genetic and biochemical analyses of the adenovirus origin of replication. The nucleotide sequence shows the first 60 base pairs of the adenovirus type 2 genome. The regions protected from DNase I by ORP-A, NF-I and ORP-C on each strand are indicated by brackets. The underlined segments represent sequences that are conserved among most human adenovirus serotypes. The bar beneath the sequence is a schematic representation of the tripartite adenovirus origin of replication defined by deletion and base substitution mutations.

41–51) contain accessory sequences that significantly increase the activity of the minimal origin. The presence of domain B increases the efficiency of initiation by more than 10-fold *in vitro*, and the presence of both domains B and C increases the efficiency of initiation by more than 30-fold. Mutations that alter the distance between the minimal origin and the accessory domains by one or two base pairs dramatically decrease initiation efficiency. This critical spacing requirement suggests that there may be specific interactions between the factors that recognize the two regions.

In contrast to the strict nucleotide sequence requirement for initiation with double-stranded templates, all single-stranded DNA molecules that have been tested support the formation of dCMP–pTP complexes with about the same efficiency (Challberg & Rawlins 1984). This finding suggests that initiation of adenovirus DNA replication occurs in two distinct steps. In the first step the template is unwound at the terminus by a mechanism that requires the recognition of specific nucleotide sequence elements by viral or cellular proteins or both. In the second step the covalent linkage between dCMP and the pTP is formed by a mechanism that requires an exposed single strand, but is relatively indifferent to sequence.

(ii) *Cellular proteins involved in initiation of adenovirus DNA replication*

The existence of several sequence domains within the adenovirus origin of DNA replication stimulated a search for the proteins that recognize them. Previous studies had demonstrated that domain B contains the recognition site for nuclear factor I (NF-I), a cellular protein that is required for optimal initiation of replication (Nagata *et al.* 1982, 1983; Rawlins *et al.* 1984; Guggenheimer *et al.* 1984; de Vries *et al.* 1985; Leegwater *et al.* 1985; Rosenfeld & Kelly 1986). We have recently identified two additional cellular site-specific DNA binding proteins, ORP-A and ORP-C, which recognize sequences in domains A and C, respectively, of the viral origin. All three proteins were purified by DNA recognition site affinity chromatography (Rosenfeld & Kelly 1986). This two-step procedure consisted of chromatography on a non-specific DNA matrix followed by chromatography on a matrix prepared from a plasmid that contains 88 copies of the adenovirus origin of replication. The boundaries of the recognition sites of the purified proteins were determined by DNase I footprint analysis and are summarized in figure 1. Analysis of the initiation efficiency of templates containing deletion

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and/or base substitution mutations in the recognition sites of NF-I and ORP-C has verified that both proteins are directly involved in initiation of adenovirus DNA replication (Rosenfeld *et al.* 1987). The status of ORP-A is not yet completely clear, owing to a lack of base substitution mutations in its recognition site. Efficient initiation has been reconstituted *in vitro* with a combination of two virus-encoded proteins and three cellular factors (figure 2). The required viral proteins are the pTP and the adenovirus polymerase, as demonstrated by previous studies (Lichy *et al.* 1982). The required cellular factors are NF-I, ORP-C, and one additional factor that has been separated from the three site-specific DNA binding proteins, but has only been partly purified.

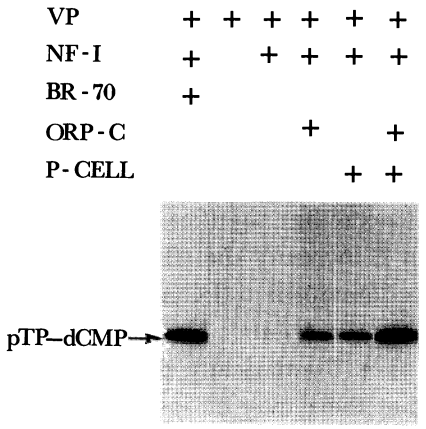

FIGURE 2. Effects of cellular factors on the initiation of adenovirus DNA replication. The preterminal protein–dCMP initiation complexes formed in the presence of [α-^{32}P]dCTP were analysed by SDS–polyacrylamide gel electrophoresis followed by autoradiography (Rosenfeld *et al.* 1987). Protein fractions were added as indicated: purified preterminal protein–DNA polymerase complex (VP); pure nuclear factor I (NF-I); a side fraction of NF-I purification that contains ORP-A, ORP-C and other cellular proteins (BR-70); pure origin recognition protein C (ORP-C); a side fraction of ORP-C purification (P-cell).

It is of interest that two of the cellular proteins that are required for optimal DNA replication *in vitro*, NF-I and ORP-C, may also play a role in cellular transcription. In collaboration with K. Jones, J. Kadonaga and R. Tjian we have found that NF-I is identical with transcription factor CTF (Jones *et al.* 1985), which appears to be responsible for selective recognition of eukaryotic promoters that contain the sequence CCAAT (Jones *et al.* 1987). Preliminary results suggest that ORP-C binds specifically to the octamer element (ATGCAAAT) that is required for optimal transcription from a variety of cellular promoters (Sive & Roeder 1986; Singh *et al.* 1986; Pruijn *et al.* 1986).

(*b*) *SV40 DNA replication* in vitro

(i) *The SV40 origin of DNA replication*

The cell-free SV40 DNA replication system is dependent upon three components: the viral initiation protein T antigen, cellular replication proteins, and a double-stranded template containing the SV40 origin of DNA replication. To define the nucleotide sequence elements required for replication *in vitro*, we measured the replication efficiencies of a large number of plasmids containing mutations in the origin region (Li *et al.* 1986*a*). These studies demonstrated that the minimal segment of viral DNA necessary for replication *in vitro* was approximately 65

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base pairs in length (map positions 5209–34) and coincided precisely with the minimal origin of replication defined by previous *in vivo* studies (figure 3). The minimal origin contains a high affinity binding site for the SV40 T antigen, a 15 base pair imperfect inverted repeat, and a 17 base pair AT-rich region. Sequences adjacent to the minimal origin, although not absolutely required, have significant effects on the efficiency of replication. The presence of a second T antigen binding site on one side of the minimal origin increases replication several fold, both *in vivo* and *in vitro*. The presence of either of two well-characterized transcriptional control elements on the other side of the minimal origin dramatically increases replication efficiency *in vivo*, but not *in vitro*. The two elements are (1) a series of GC-rich repeats that bind the transcription factor SP1 and (2) a pair of 72 base pair transcriptional enhancer elements. It has previously been shown that both of these transcriptional elements are associated with a change in local chromatin structure that renders the adjacent DNA deficient in nucleosomes and accessible to nucleases (Fromm & Berg 1983; Gerard *et al.* 1985; Jongstra *et al.* 1984). Thus they may increase replication efficiency by facilitating the access of initiation factors to the replication origin.

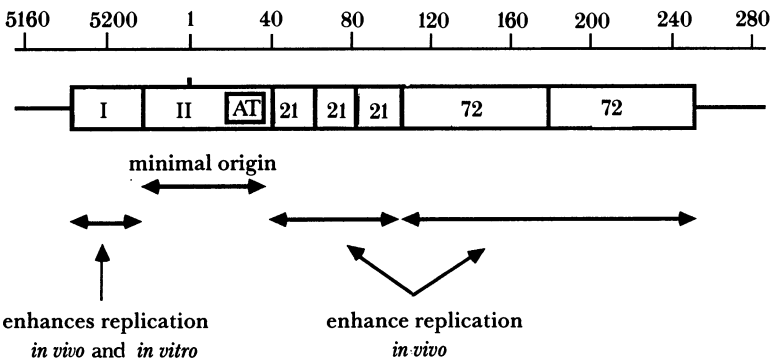

FIGURE 3. SV40 sequences required for DNA replication *in vitro* and *in vivo*. The diagram shows the sequence organization of the SV40 origin region. The following elements are indicated: I represents T antigen binding site I; II represents T antigen binding site II; AT is the 17 base pair AT-rich region; 21 is the 21 base pair repeats containing the binding sites for transcription factor SP1; 72 represents the 72 base pair repeats containing the SV40 transcriptional enhancers.

(ii) *The protein requirements for SV40 DNA replication*

We are currently attempting to exploit the cell-free SV40 replication system to examine the roles of viral and cellular proteins in the replication process. The general approach to identifying required replication proteins has involved fractionation of the crude extract by various methods and reconstitution of replication activity with separated fractions. Our data indicate that optimal DNA replication *in vitro* requires at least four distinct protein components.

(1) SV40 T antigen. The T antigen is the only virus-encoded protein required for SV40 DNA replication. As described in greater detail below, the helicase activity of T antigen (Stahl *et al.* 1986) appears to be crucial for initiation of replication.

(2) Cellular fraction I. This fraction is devoid of polymerase–primase activity and contains a component(s) required before the elongation phase of SV40 DNA synthesis (see below).

(3) Cellular fraction II. This fraction contains the cellular DNA polymerase α with its associated primase activity and perhaps other required proteins as well.

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(4) Cellular DNA topoisomerase. Topoisomerases play important roles during two different steps in the replication of closed circular templates. During the chain elongation phase a topoisomerase is required to catalyse the progressive decrease in linking number of the parental strands that must accompany the advancement of the replication forks. Either of the two known cellular topoisomerases (topoisomerase I or topoisomerase II) is sufficient to provide this unlinking activity. Topoisomerase activity is also required for the segregation of the newly synthesized daughter molecules upon completion of DNA synthesis. Only the cellular topoisomerase II can perform this function (Yang *et al.* 1987).

(iii) *Initiation of SV40 DNA replication*

The kinetics of DNA replication in the cell-free system is complex. Under the standard reaction conditions there is an initial period of 15–20 min during which DNA synthesis is almost undetectable. DNA synthesis then increases rapidly and reaches a constant rate, which is maintained for at least two hours. These results suggest that one or more slow steps must occur before rapid chain elongation can take place. In support of this view we found that the initial lag-phase of the reaction can be eliminated by preincubation of the DNA template with T antigen, cellular fraction I, and ATP (Li *et al.* 1986*b*). The recent discovery that T antigen has helicase activity (Stahl *et al.* 1986) prompted us to ask whether origin-dependent unwinding of the template might be occurring during this presynthesis reaction. When the presynthesis reaction was done in the presence of HeLa cell topoisomerase I, a novel DNA species was formed that migrated with an electrophoretic mobility similar to that of form I SV40 DNA (figure 4). The protein requirements for formation of this species were the same as those for removal of the presynthetic lag, and the reaction was specific for templates that contain the wild-type SV40 origin of DNA replication. We have identified the rapidly migrating species as a highly underwound form of the template by examination in the electron microscope. Under conditions where control templates are fully duplex, the underwound species exhibits extensive single-stranded regions. Thus the underwound species is similar to structures observed during the prepriming phase of two prokaryotic replication systems, namely *Escherichia coli* and bacteriophage lambda (Baker *et al.* 1986; Dodson *et al.* 1986). We have also found that an underwound species is formed when *E. coli* single-stranded DNA binding protein (SSB) is substituted for cellular fraction I during the presynthesis reaction (figure 4*c*). This finding indicates that a major active component of cellular fraction I may be a eukaryotic analogue of SSB.

Our data provide strong support for the hypothesis that the first step in initiation of SV40 DNA replication involves the specific binding of T antigen to the origin followed by the T antigen-mediated unwinding of the DNA in the origin region. The reaction is specific for the wild-type origin, requires ATP hydrolysis, and may be facilitated by other proteins, including a protein with properties similar to prokaryotic SSB. This hypothesis is consistent with the work of Stahl *et al.* (1986) who first demonstrated that T antigen possessed an intrinsic helicase activity dependent upon ATP. The T antigen helicase activity is capable of completely unwinding an oligonucleotide hybridized to a long single strand (Stahl *et al.* 1986) and will partly unwind even completely duplex circular DNA (Wold *et al.* 1987). The postulated role of T antigen in initiation is also consistent with previous work indicating that both the specific DNA binding activity and the ATPase activity of the protein are required for SV40 DNA replication (Clark *et al.* 1983; Paucha *et al.* 1986; Cole *et al.* 1986; Smale & Tjian 1986).

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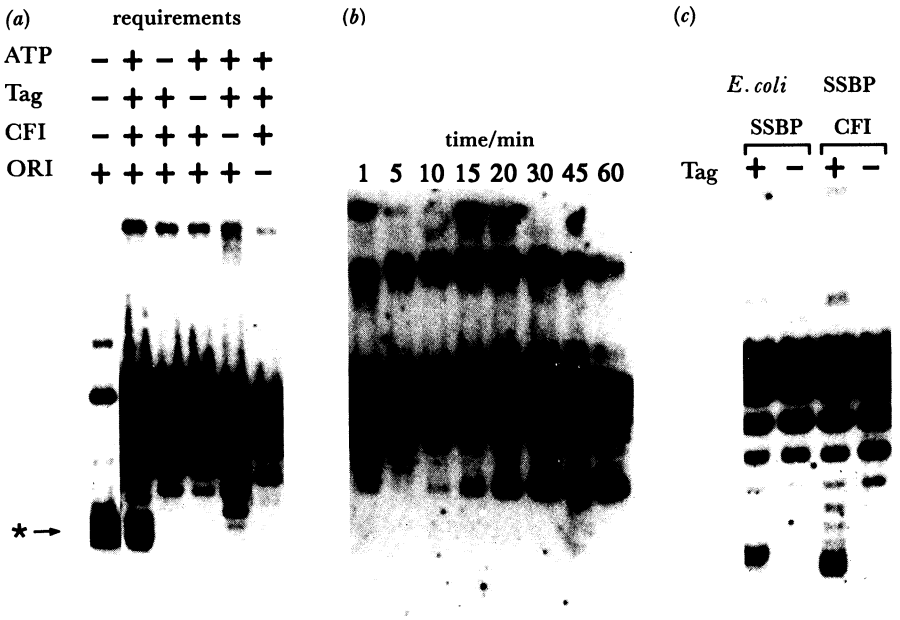

FIGURE 4. Specific DNA unwinding during the presynthesis reaction. Presynthesis reaction mixtures containing T antigen (Tag), cellular fraction I, ATP, topoisomerase I and a plasmid DNA template were incubated 20–30 min at 37 °C except where indicated otherwise (Wold *et al.* 1987). (*a*) Requirements for the reaction. The DNA templates were pOR.HSO, which contains the wild-type SV40 origin (ori+) or pOR.8-4, which is identical to pOR.HSO except for a four base pair deletion in the origin (ori−). ★, Position of the unwound species of DNA. (*b*) Time-course of the reaction with pUC.HSO as template. The numbers refer to time of incubation in minutes. (*c*) Unwinding of templates with SSB. Reactions contain either SSB (SSBP) or cellular factor I (CFI) as indicated.

Finally, the results suggest similarities in the initiation mechanisms operative during prokaryotic and eukaryotic DNA replication. It would appear that the first steps in replication may be quite similar in the two cases and that T antigen combines in a single protein the functions attributed to several different prokaryotic proteins.

CONCLUSIONS AND SPECULATIONS

Although there are many obvious differences in the mechanisms operative during the replication of adenovirus and SV40 DNA, there are also some fundamental similarities. In both cases it is likely that the first step in replication involves the unwinding of the parental strands in the region of the origin of DNA replication (figure 5). It is also likely that specificity of the initiation reaction is determined at this unwinding step. In both systems the site of initiation is controlled by the specific interaction of one or more proteins with sequence elements in the origin. The evidence available at this time suggests that cellular proteins are the primary determinants of specificity in the adenovirus system. In the SV40 system the virus-encoded T antigen plays a central role in origin recognition and unwinding; however, given the complexity of the SV40 origin region it seems probable that cellular DNA binding proteins may be involved as well.

The nature of the priming event required to initiate daughter strand synthesis appears to be quite different for adenovirus and SV40. In the case of adenovirus a virus-encoded protein, the

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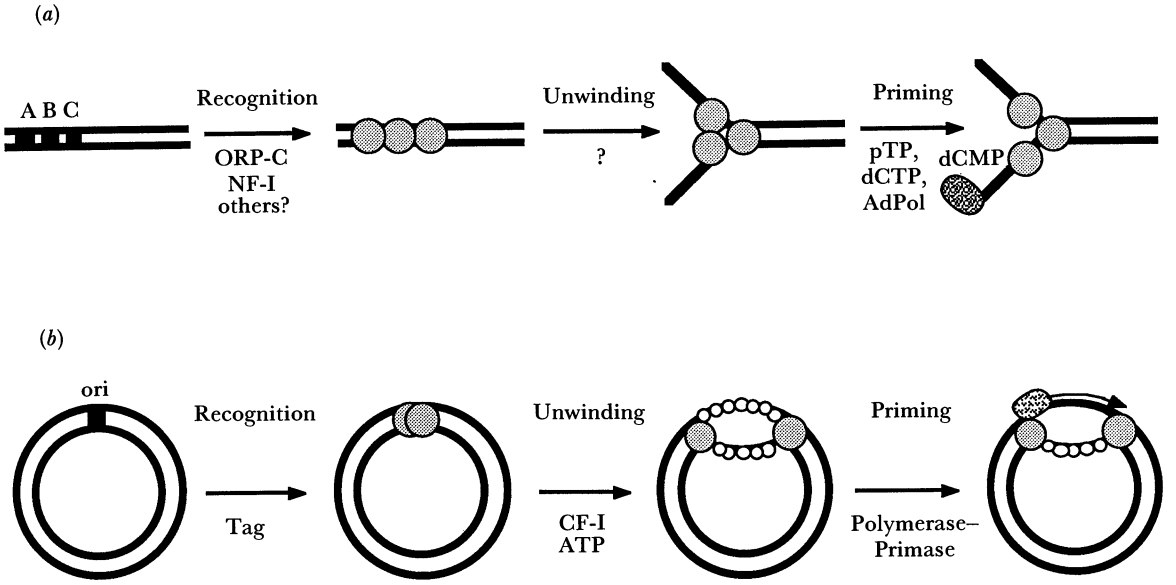

FIGURE 5. Model of initiation of replication of (*a*) adenovirus and (*b*) SV40 DNA.

preterminal protein, serves as primer in a reaction that also requires the participation of the viral polymerase (Lichy *et al.* 1982). Although the initial priming event in SV40 DNA replication has not yet been studied in detail *in vitro*, analysis of replication *in vivo* strongly suggests that an RNA primer synthesized by the cellular primase–polymerase complex is involved (Hay & DePamphilis 1982). In spite of the apparent difference in mechanism, these two priming reactions do have some general features in common. Both appear to operate on single-stranded DNA templates, and, except for a preference for the initiating nucleotide, both display rather limited sequence specificity.

Finally, there is evidence in both the adenovirus and the SV40 systems for the involvement of protein factors and sequence elements that play a role in cellular transcription. Recent studies suggest that some of the cellular proteins that bind specifically to the adenovirus origin also facilitate the transcription of certain cellular promoters. Moreover, it has been demonstrated that sequence elements involved in transcription of the SV40 genome (the GC-rich repeats and enhancers) can significantly enhance the replication of SV40 DNA *in vivo*. These data suggest that there may be common features to transcription and replication in animal cells, and that a given site-specific DNA binding protein may participate in both processes.

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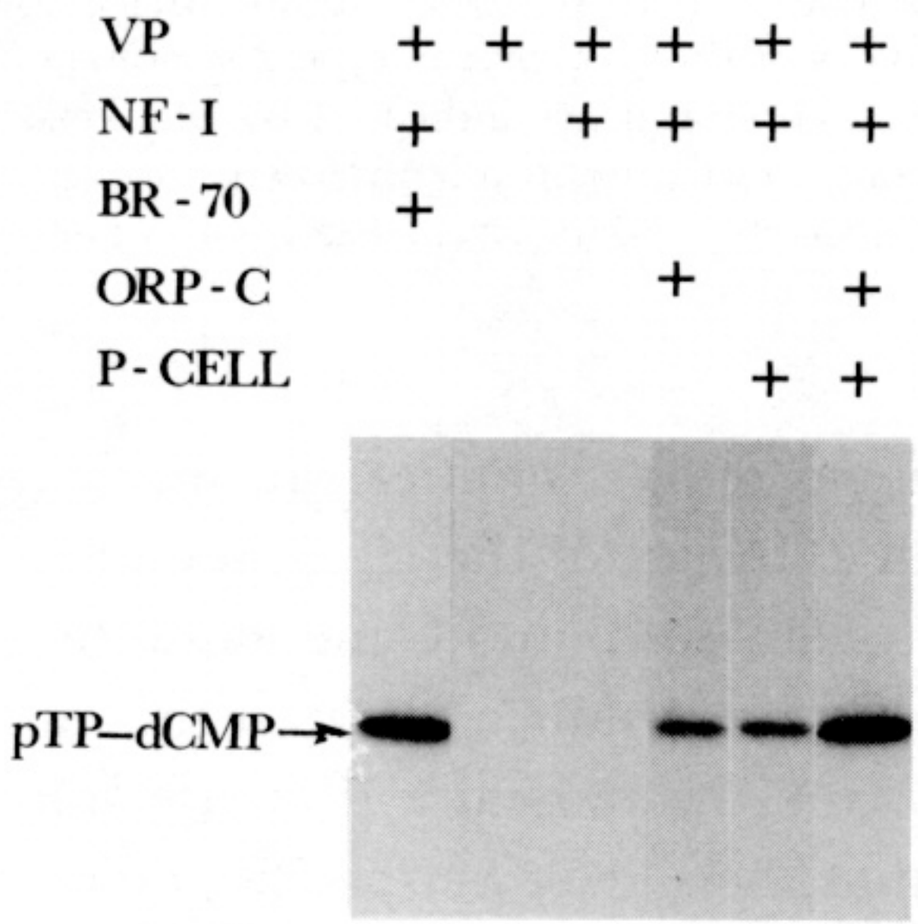
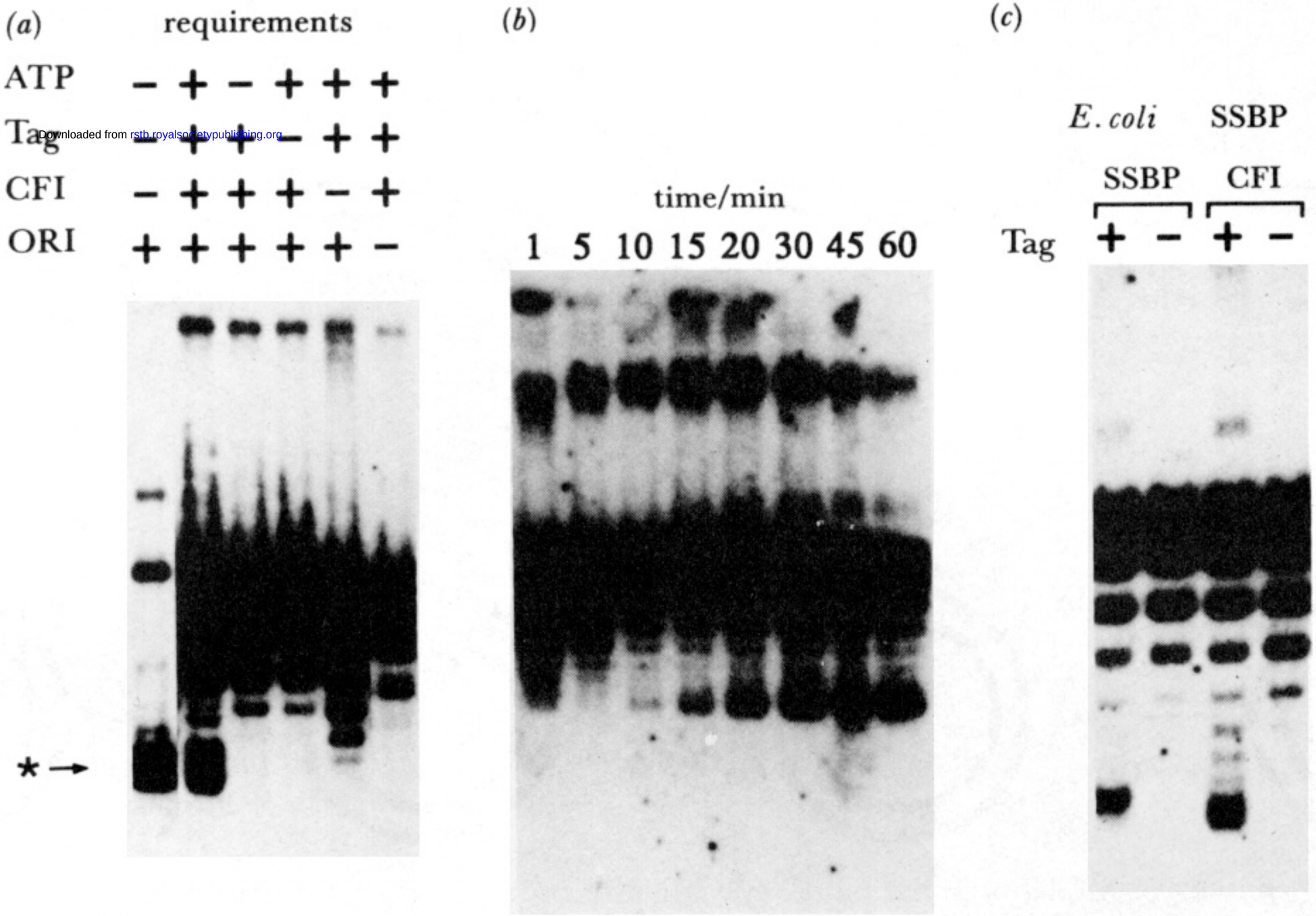
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



GURE 2. Effects of cellular factors on the initiation of adenovirus DNA replication. The preterminal protein–dCMP initiation complexes formed in the presence of [α-^{32}P]dCTP were analysed by SDS–polyacrylamide gel electrophoresis followed by autoradiography (Rosenfeld *et al.* 1987). Protein fractions were added as indicated: purified preterminal protein–DNA polymerase complex (VP); pure nuclear factor I (NF-I); a side fraction of NF-I purification that contains ORP-A, ORP-C and other cellular proteins (BR-70); pure origin recognition protein C (ORP-C); a side fraction of ORP-C purification (P-cell).



GURE 4. Specific DNA unwinding during the presynthesis reaction. Presynthesis reaction mixtures containing T antigen (Tag), cellular fraction I, ATP, topoisomerase I and a plasmid DNA template were incubated 20–30 min at 37 °C except where indicated otherwise (Wold *et al.* 1987). (*a*) Requirements for the reaction. The DNA templates were pOR.HSO, which contains the wild-type SV40 origin (ori+) or pOR.8-4, which is identical to pOR.HSO except for a four base pair deletion in the origin (ori−). ★, Position of the unwound species of DNA. (*b*) Time-course of the reaction with pUC.HSO as template. The numbers refer to time of incubation in minutes. (*c*) Unwinding of templates with SSB. Reactions contain either SSB (SSBP) or cellular factor I (CFI) as indicated.